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(54) Title: IMPROVED ALGINATE MICROCAPSULES, METHODS OF MAKING AND USING SAME

(57) Abstract

A composition for containing biological material for *in vivo* implantation and transplantation comprising alginate cross-linked with barium salt, preferably barium chloride. The microcapsule may optionally have as its other layers hyaluronic acid and poly-L-lysine. Also alginate cross-linked with both calcium chloride and barium chloride may be used. The microcapsule of the present invention is rugged and retains a strong negative charge, enhancing the release of protein and limiting the invasion of immunoglobulins. The microcapsule may preferably be used for encapsulating islets of Langerhans for the production of insulin.

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DESCRIPTION

Improved Alginate Microcapsules, Methods of Making and Using Same

This is a continuation of co-pending application U.S. Serial No. 07/449,983, filed December 13, 1989.

Background of the Invention

1. Field of the Invention

This invention relates to the fields of polymer chemistry, immunology and transplantation, and more particularly to the field of materials for use in conjunction with transplantation and implantation of foreign cells and biological materials.

2. Art Background

Evidence exists that transplantation of insulin-producing cells (islets) can cure the diabetic animal of the need for insulin therapy. The major obstacle preventing clinical success in islet transplantation as a therapy for diabetes to date has been immunogenicity of the cell and rejection of the transplanted graft. Survival of islet allografts and or xenografts has been achieved by various methods of immunosuppression and/or related immunological techniques. However, such techniques have had only limited success in that the transplanted islet cells survive only a short while before rejection occurs. In addition, the extended use of immunosuppressive agents often results in severe complications, such as renal damage and even cancer in the transplant recipient.

One solution to this problem of graft rejection is the introduction of a physical, semi-permeable barrier between the transplanted islets and the host's immune system by the method of microencapsulation. Microencapsulation is a process in which small, discrete

materials, viable biological tissue or cells, liquid droplets, or gases are completely enveloped by an intact membrane which is preferably compatible with the biological system in which it is placed. The function of the microcapsule membrane is to protect the material within from immunological recognition by the host and to control the flow of materials inside and outside the microcapsule across the membrane.

A large body of literature on microencapsulation has been produced including Darquy, S. and Reach, G. Diabetologia, (1985) 528:776-780; Lim, F. and Sun, A. Science, (1980) 210:908-910; Lim, F. and Moss, R. Journal of Pharmaceutical Sciences. (April, 1981) 351 - 354; O'Shea, et al. Biochemica et Biophysica Acta. 804 (1984) 133- 136; Leung, et al. Artificial Organs. (1983) 7(2) 208-21 2; Araki, et al. Diabetes, Vol. 34, September 1985, 850-854; and U.S. Patent Nos. 4,690,682; 4,409,331; 4,391,909, among others.

In addition to islet cells, other materials such as microbial cells, other mammalian cells, yeasts, chloroplasts, plant protoplasts, mitochondria and enzymes have been immobilized and entrapped using microencapsulation techniques.

Among the materials used in encapsulation are calcium alginate gels. Lim and Sun, in 1980, successfully micro-encapsulated islets using alginate gel, poly-L-lysine and polyethylenimine. The encapsulated islets were injected intraperitoneally into diabetic rats. The animals' blood glucose levels dropped to normal for two to three weeks, suggesting that the encapsulation process had protected the islets from invasion by the recipients' immune system. However, these studies showed that the microcapsules were eventually rejected as a result of fibrosis, or fibroblast formation around the microcapsule, which eventually restricts the flow of nutrients to the cells contained in the microcapsule and the outflow of material from the microcapsules created by the islet cells disposed therein.

The Lim and Sun capsules are usually made by first forming a negatively charged alginate bead around purified and isolated islet cells by cross-linking alginate with calcium chloride, then creating a positively charged membrane on the outer surface by forming an ionic bond with a cation such as poly-L-lysine. Additionally, a second negatively charged outer layer of alginate is usually formed around the outside of the poly-L-lysine layer, ionically bonded thereto. Finally, the inner bead of alginate is degelled, leaving a capsule surrounded by a layer of poly-L-lysine-alginate gel and an outer layer of alginate. This prior art capsule is depicted in Figure 1 and described in more detail below.

Capsules formed according to the foregoing procedure are difficult to make, requiring many steps, which is not advantageous in light of the consideration that live cells are involved. Also, it is desirable to minimize handling time and moderate handling conditions. Even more significant, however, is the fact that these prior art capsules often fail in vivo as a result of the release of substances which stimulate cytokine release, which in turn cause the microcapsules to be attacked by immunoglobulins. The immunoglobulins may either, or in combination, penetrate the microcapsule and destroy the enclosed islet cells, cause fibroblast formation around the microcapsule thereby choking off nutrients to the cells and preventing the cell products from being released into the host; and/or stimulate the destruction of the microcapsule via the host's immunological system.

Alginate, the principal material of the microcapsules, is a heterogeneous group of linear binary copolymers of 1-4 linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). The monomers are arranged in a blockwise pattern along the polymeric chain where homopolymeric regions ar interspaced with sequences containing both monomers. The proportion and sequential arrangement of the uronic acids in alginate depend upon

the species of algae and the kind of algal tissue from which the material is prepared. Various properties of different types of alginates are based upon the guluronic acid makeup of the particular alginate. For example, viscosity depends mainly upon the molecular size, whereas the affinity for divalent ions essential for the gel-forming properties are related to the guluronic acid content. Specifically, two consecutive di-axially linked G residues provide binding sites for calcium ions and long sequences of such sites form cross-links with similar sequences in other alginate molecules, giving rise to gel networks.

It has been demonstrated that a significant stimulant to the release of cytokines is the 1-4 linked β-D-mannuronic acid (M) component of alginate. (See copending patent application Serial No. 468,905. These M blocks do not bind with calcium when the gel is formed in the inner bead, and it is believed that some of this M alginate leaches out after the microcapsule is formed.

In accordance with the theories in the prior art, it has traditionally been believed that microcapsules form an effective barrier against immunoglobulin penetration by having a sufficiently small diameter porosity that large proteins are excluded. However, it may be that the negative charge of the alginate bead plays a more significant role in excluding negatively charged proteins, such as immunoglobulins.

The present invention overcomes the deficiencies of the prior art by providing a microcapsule of the type described herein in accordance with the following description, as well as ancillary materials and methods relating thereto.

Summary of the Invention

The present invention provides a successful approach to microencapsulation and implantation which has not heretofore been discovered.

It is one object of the present invention to provide a material which may be implanted or transplanted in vivo which is non-immunogenic and non-fibroblast inducing.

It is yet another object of the present invention to provide a microencapsulation system utilizing alginate which is gelled using barium salt instead of the prior art calcium chloride.

It is another object of the invention to provide a microencapsulation system in which the alginate bead remains in a gelled state.

It is yet another object of the present invention to provide a microcapsule which is more rugged and durable than prior art capsules, and which retains a greater negative charge over a longer period of time than prior art microcapsules.

It is another object of the present invention to provide a microencapsulation system which decreases immunogenicity relative to prior art capsules by limiting the leaching of M block alginate.

Still another object of the present invention is to provide a microencapsulation system which increases immunoprotectability of the contents thereof by increasing and maintaining the negative charge in the core, thereby preventing or minimizing the entry into said microcapsule of negatively charged immunoglobulins.

Yet another object of the present invention is to provide a microencapsulation system with improved insulin or other protein or product release characteristics resulting from the negative charge in the capsule.

The present invention comprises a new encapsulation material comprised of alginate gelled by barium salt, the material being useful in vivo for implantation and transplantation in mammalian bodies. The material may take many forms, such as sheets, organ capsulation and the like, but is preferably used for microencapsulation of living cells which are foreign to the host in which they are implanted. The present invention also protects the

islets of Langerhans or other transplanted tissue from immunological cell rejection. The present invention also provides a microencapsulation system which limits fibroblast overgrowth.

In one embodiment, the present invention relates to encapsulation of cells or other biological material with a coating of alginate gelled with barium salt, preferably, barium chloride. Optionally, a second layer of poly-L-lysine, and a third outermost layer of alginate, may be added to the capsule. The alginate in the outer coating is preferably comprised of substantially guluronic acid, with minor amounts of mannuronic acid blocks.

In a second embodiment, the alginate portion of either of the former embodiments is gelled with a combination of barium and calcium.

In yet another embodiment, the inner layer of the microcapsule is comprised of barium gelled or barium plus calcium gelled alginate, which is then coated with a poly-L-lysine and an outer layer of hyaluronic acid.

Other embodiments, and the details of the present invention will be best understood with reference to the drawings provided herewith and described briefly below.

Brief Description of the Drawings

FIGURE 1 is an illustration of a cross-section of the prior art microcapsule depicting the various layers and one example of the potential contents of the microcapsule.

FIGURE 2 is an illustration of a cross-section of one embodiment of the present invention.

FIGURE 3 is an illustration of a cross-section of another embodiment of the present invention.

FIGURE 4 is an illustration of a cross-section of the preferred embodiment of the present invention.

FIGURE 5 is an illustration of a cross-section of another embodiment of the present invention.

Detailed Description of the Invention

The present invention comprises material which can be implanted or transplanted in vivo into mammals without inducing any substantial immunogenic reaction or fibroblast formation. The present invention also comprises materials for encapsulation of biological materials. The present invention is also a process for microencapsulating biological cells and other materials for use in implantation or transplantation as a drug or biological material delivery system. As used herein, the term biological materials includes prokaryotic and eukaryotic cells which are either naturally occurring or genetically engineered, drugs or pharmaceuticals, enzymes, parts of cells such as mitochondria and protoplasts or any other naturally occurring or synthesized material which may be implanted.

The material used in the present invention is alginate cross-linked with barium salt, and preferably barium chloride. The alginate may be any alginate solution capable of forming microcapsules, as is known in the art. The alginate may be comprised substantially of α-L-guluronic acid (G) which may be referred to herein as guluronic acid alginate or high G.

The use of high guluronic acid alginate is described in our copending patent application Serial No. 446,462. Small amounts of mannuronic acid (β-D-mannuronic acid) are also present. There are at least 65% G residues or more, and preferably about 85% G residues and 15% or less M residues in high G alginate. Alginate so comprised elicits a very low response from monocytes in the production of tumor necrosis factor (TNF) and IL-1 and IL-6, and, as a result, does not elicit fibrosis. Such alginate may be obtained from Protan A/S, Drammen, Norway. High G alginate is the preferred alginate used on the outside of microcapsules because of its property of not inducing fibroblast formation.

Figure 1 shows the prior art capsule of Lim and Sun. As shown in Figures 1, such prior art microcapsules comprise islets of Langerhans 12 or other substance for transplantation or implantation contained in a liquid bead or capsule of alginate 14 which was gelled with calcium chloride during the making of the microcapsule and then ungelled to return it to a liquid state. Surrounding the calcium-alginate liquid bead is a layer of poly-L-lysine 16 which forms a membrane by bonding ionically with the alginate core. On the outside is another layer of alginate 20.

As shown in Figure 2, the present invention, in one embodiment, comprises islets of Langerhans 12 or other transplantation or implantation material, coated with a bead of alginate 22 gelled with a barium salt, preferably barium chloride.

As shown in Figure 3 the islet of Langerhans 12 may be surrounded by a barium alginate gel coating 22, as in Figure 2, which in turn is surrounded by a poly-L-lysine layer 16, which in turn is surrounded by an outer layer of alginate 24, preferably high G alginate.

As shown in Figure 4, which depicts the preferred embodiment, the islet of Langerhans 12 may be surrounded by an alginate gel coating 26, that is gelled with both calcium and barium, which in turn is surrounded by a poly-L-lysine layer 16, which in turn is surrounded by an outer layer of alginate 24, preferably high G alginate.

As shown in Figure 5 the islet of Langerhans 12 may be surrounded by a barium alginate bead 22 or an alginate bead gelled with both calcium and barium 26, which in turn is surrounded by a poly-L-lysine layer 16, which in turn is surrounded by an outer layer of hyaluronic acid 30.

Thus, the barium alginate capsule may be used alone or in conjunction with other layers to form a microcapsule. In the preferred embodiment, a 1.0% to 1.5% by weight alginate solution is formed around purified islets f Langerhans and is treated with a solution in the

range of 2 to 20 mM barium chloride to form a gelled microcapsule.

In another embodiment, an alginate bead having a concentration of 1.0 to 1.5% alginate is first treated with a solution of 80 to 100 mM calcium chloride, to bind the G-blocks, and then with a second solution of 1 to 20 mM barium chloride to bind the blocks of the alginate composition.

In yet another embodiment, the microcapsule of the immediately foregoing embodiment is further treated with a solution of 0.5% poly-L-lysine (20,000 MW). An outer coating of 1.1% alginate, preferably high G alginate, is then formed therearound.

As a middle layer, poly-L-lysine is the preferred material. However, it will be appreciated by a person of ordinary skill in the art that poly-L-ornithine and chitosan may be used in place of poly-L-lysine, and that other cationic compounds with similar properties may also be used.

The use of hyaluronic acid as a component of the present invention inhibits the formation of fibroblasts when applied as an outside coating on the microcapsule.

There are many improvements provided as a result of the present invention. First, barium alginate tends to be a more rugged and hardy material than prior art calcium alginate. Also, fewer steps are required in the manufacture of barium alginate microcapsules, first because multiple layers are not required, and also, if as many layers are used, there is no need for a de-gelling step as is used in the prior art.

When alginate beads are treated with both barium chloride and calcium chloride, the bead is first dropped in a solution of calcium chloride, and then in a solution of barium chloride. The calcium is believed to cross-link with the guluronic acid blocks of he alginate molecules, and the barium cross-links both with the M-block portions

of the alginate and the G-block portions which have not previously been cross-linked with the calcium chloride.

The resulting microcapsules of the present invention have improved kinetics of insulin release. The barium chloride gel material has a greater negative charge because it is in a gel form, rather than a liquid form, and also because over time, the liquid calcium alginate of the prior art microcapsules leaches out so there is less negatively charged material in prior art microcapsules. The negatively charged portion repels the negatively charged insulin, or other negatively charged material thereby forcing said insulin or other material out of the microcapsule. Conversely, the negative charge would also repel immunoglobulin molecules produced by the host, thereby safely protecting the contents of the microcapsule.

For in vivo applications of the present invention, the composition comprising alginate having a high G content may be used in the form of organ capsulation, sheets of alginate for implantation, hollow fibers and membranes formed of the subject composition.

Example 1

Single-layer Microencapsulation of Islets of Langerhans

Cultured rat islets of Langerhans (2 X 10^3 islets in 0.2 ml medium) may be suspended uniformly in 2 ml of a 1.5% (w/w) sodium alginate solution (viscosity 51 cps) in physiological saline. Spherical droplets containing islets were produced by syringe pump/air jet extrusion through a 22-gauge needle and collected in 1.5% (w/w) barium chloride solution. The supernatant was decanted and the gelled spherical alginate droplets, containing islets, were washed with dilute CHES (2-cyclohexylamino-ethane sulfonic acid) solution and 1.1% barium chloride solution.

The microcapsules are found to be generally spherical and each to contain from 1 to 2 viable islets. The

microcapsules have a diameter of 500 ± 50 μm and wall thicknesses of about 3-4 μm. The microcapsules may be suspended in nutrient medium at 37°C.

Example 2

Mutliple-layer Microencapsulation of Islets of Langerhans

Cultured rat islets of Langerhans (2 X 10^3 islets in 0.2 ml medium) may be suspended uniformly in 2 ml of a 1.5% (w/w) sodium alginate solution (viscosity 51 cps) in physiological saline. Spherical droplets containing islets were produced by syringe pump/air jet extrusion through a 22-gauge needle and collected in 1.5% (w/w) barium chloride solution. The supernatant was decanted and the gelled spherical alginate droplets, containing islets, were washed with dilute CHES solution and 1.1% barium chloride solution.

After aspirating off the supernatant, the gelled droplets were incubated for 6 minutes in 0.05% (w/w) polylysine having a molecular weight of 17,000.

The supernatant was decanted and the polylysine capsules were washed with dilute CHES, 1.1% calcium chloride solution and physiological saline. The washed polylysine capsules were incubated for 4 minutes in 30 ml of 0.03% sodium alginate to permit he formation of an outer alginate membrane on the initial polylysine membrane, by ionic interaction between the negatively charged alginate and the positively charged polylysine. The alginate used in the outer coating, and if desired, the inner coating as well, is poly G alginate (Protan) produced as described above.

The microcapsules are found to be perfectly spherical and each to contain from 1 to 2 viable islets. The microcapsules have a diameter of 700 ± 50 μm and wall thicknesses of about 5 μm. The microcapsules may be suspended in nutrient medium at 37°C.

Example 3

Barium-Calcium Alginate Microencapsulation of Islets of Langerhans

Cultured rat islets of Langerhans (2 X 10^3 islets in 0.2 ml medium) were suspended uniformly in 2 ml of a 1.5% (w/w) sodium alginate solution (viscosity 51 cps) in physiological saline. Spherical droplets containing islets were produced by syringe pump/air jet extrusion through a 22-gauge needle and collected in 1.5% (w/w) calcium chloride solution. The supernatant was decanted and the gelled spherical alginate droplets, containing islets, were then collected in 1.5% (w/w) barium chloride. The supernatant was again decanted and the gelled spherical alginate droplets were washed with dilute CHES solution and 1.1% calcium chloride solution.

After aspirating off the supernatant, the gelled droplets were incubated for 6 minutes in 0.05% (w/w) polylysine having a molecular weight of 17,000.

The supernatant was decanted and the polylysine capsules were washed with dilute CHES, 1.1% calcium chloride solution and physiological saline. The washed polylysine capsules were incubated for 4 minutes in 30 ml of 0.03% sodium alginate to permit the formation of an outer alginate membrane on the initial polylysine membrane, by ionic interaction between the negatively charged alginate and the positively charged polylysine. The alginate used in the outer coating, and if desired, the inner coating as well, is poly G alginate produced as described above.

The microcapsules are found to be perfectly spherical and each to contain from 1 to 2 viable islets. The microcapsules would have a diameter of 700 ± 50 μm and wall thicknesses of about 5 μm. The microcapsules may be suspended in nutrient medium at 37° C.

It will be obvious to a person of ordinary skill in the art that the present invention is not limited in its application to specific biological materials to be

encapsulated, such as the islet cells described in detail above, or by the specifically described other inner layers of microcapsule discussed herein. The only limitations of the present invention are set forth in the claims appended hereto and any equivalents thereof.

Claims

1. A transplantation or implantation material comprising biological material encapsulated with a coating material comprising alginate cross-linked with barium salt.

2. The material of Claim 1 wherein said biological material comprises purified islets capable of producing insulin.

3. The material of Claim 1 wherein said biological material is microencapsulated in a first innermost layer of barium chloride cross-linked alginate, a second intermediate layer of cationic material selected from poly-L-lysine, poly-L-ornithine and chitosan and a third outermost layer selected from alginate comprised substantially of α-L-guluronic acid with minor amounts β-D-mannuronic acid and hyaluronic acid.

4. The material of Claim 3 wherein, said second layer is poly-L-lysine and said third layer is alginate comprised substantially of gelled α-L-guluronic acid with minor amounts β-D-mannuronic acid.

5. The composition of Claim 4 wherein said poly-L-Lysine comprises a molecular weight of less than 20,000 daltons.

6. The composition of Claim 1 wherein said alginate is cross-linked with barium chloride and calcium chloride.

7. A method of encapsulating biological material comprising forming around said biological material a bead of alginate gel cross-linked with barium salt.

8. The method of Claim 7 wherein said biological material comprises islet cells.

9. The method of Claim 8 wherein said islet cells are first microencapsulated in a bead of barium cross-linked alginate gel, then encapsulated in a layer composed of material selected from poly-L-lysine, poly-L-ornithine and chitosan, and then encapsulated in an outermost layer comprising material selected from hyoluronic acid and alginate comprised substantially of α-L-guluronic acid with minor amounts β-D-mannuronic acid.

10. The method of Claim 9 wherein said second layer material is poly-L-lysine and said outermost layer material is alginate comprised substantially α-L-guluronic acid with minor amounts β-D-mannuronic acid.

11. The method of Claim 10 wherein said poly-L-Lysine comprises a molecular weight of less than 20,000 daltons.

12. The method of Claim 7 further comprising cross-linking said first layer of alginate with both barium salt and calcium salt.

13. A method of making a microcapsule for containing islets of Langerhans comprising forming around said islets of Langerhans a bead comprised of alginate, and gelling said alginate with barium chloride.

14. The method of Claim 13 wherein said alginate is in a concentration in the range of 1% to 1.5%.

15. The method of Claim 13 wherein said barium chloride in the range of 2 to 20 mM.

16. The method of Claim 13 further comprising treating said microcapsule with calcium chloride in the range of 80 to 100 mM.

FIG. 1.

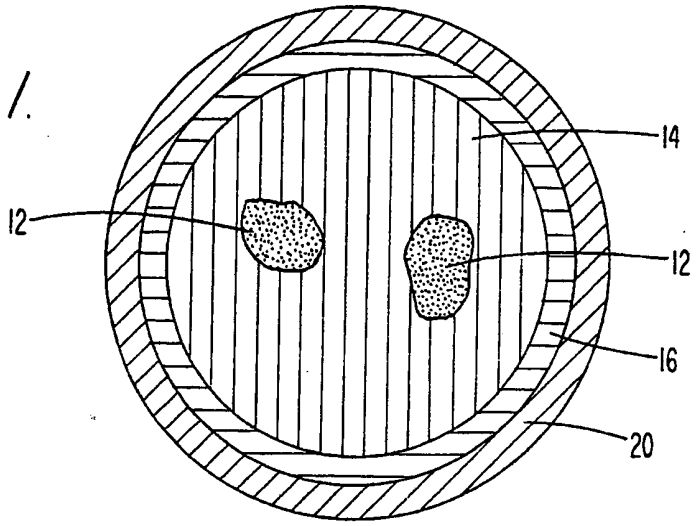


FIG. 2.

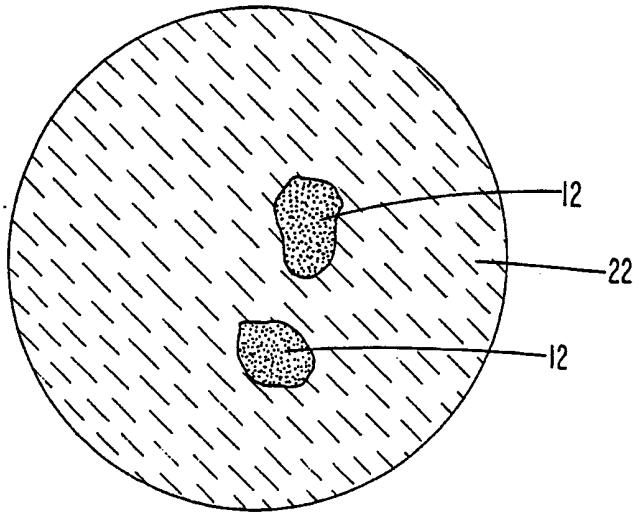


FIG. 3.

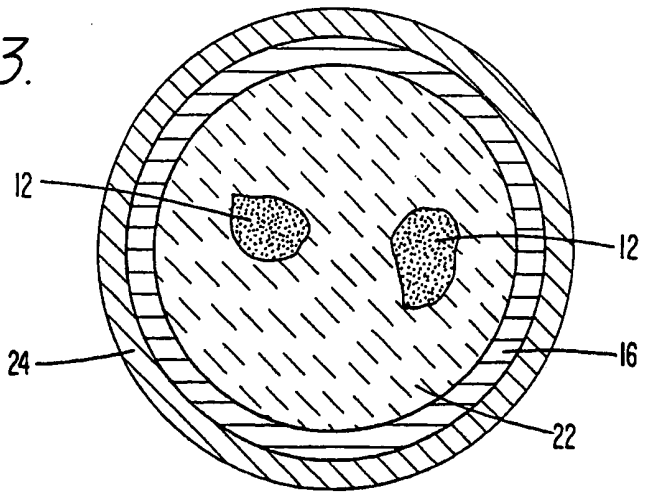


FIG. 4.

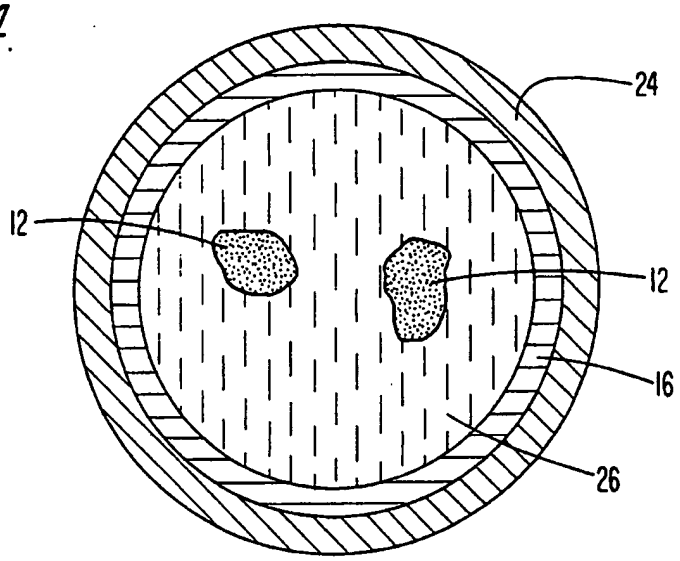


FIG. 5a.

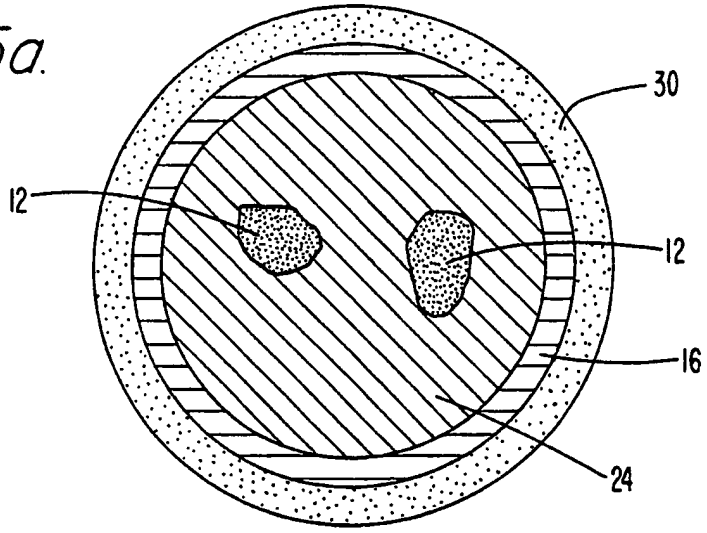
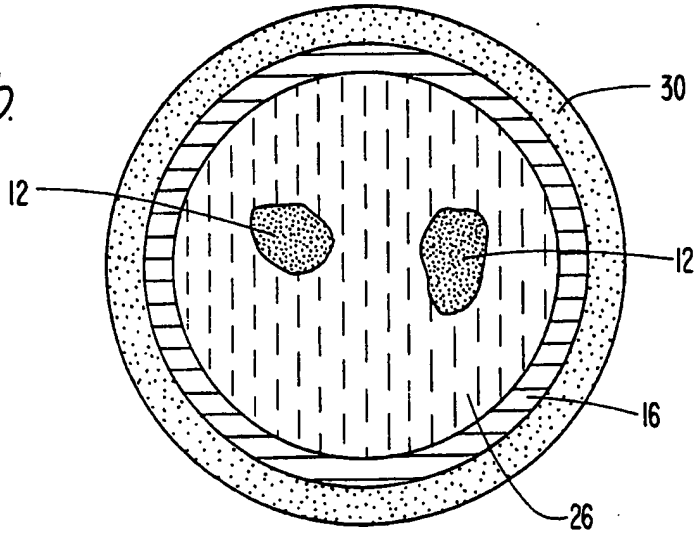


FIG. 5b.



INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/07275

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 11/10, 11/04, 5/00
U.S.Cl.: 435/178, 182, 240.22

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/178, 179, 182, 240.22; 436/529; 530/813; 536/3

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X, P Y	US,A, 4,950,600 (Tanaka et al.) 21 August 1990, see entire document.	1, 7 2-6, 8-16
Y	US,A, 4,352,883 (Lim) 05 October 1982, see entire document.	1-16
Y	US,A, 4,407,957 (Lim) 04 October 1983, see entire document.	1-16
A	US,A, 4,663,286 (Tsang et al.) 05 May, 1987, see entire document.	

* Special categories of cited documents:
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 February 1991	22 MAR 1991
International Searching Authority: ISA/US	Signature of Authorized Officer: David M. Naff

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